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NOVEL PROCOAGULANT PROTEINS

This invention relates to a novel series of proteins which exhibit procoagulant properties. These proteins have marked structural differences from human factor VIII:C, but have similar procoagulant activity.

Factor VIII:C is the blood plasma protein that is defective or absent in Hemophilia A disease. This disease is a hereditary bleeding disorder affecting approximately one in 20,000 males. The structure of factor VIII:C is described in U.S. Patent Applications Serial No. 546,650 filed October 28, 1983 and No. 644,036 filed August 24, 1984, which are incorporated herein by reference. This is further confirmed in Nature, 312:306, 307, 326 and 342.

Brief Summary of the Drawing

Figure 1 illustrates the DNA nucleotide sequence of one strand only coding for the complete human factor VIII:C and the deduced amino acid sequence.

Detailed Description

This invention provides for proteins which have procoagulant

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activity similar to that of factor VIII:C and also have substantially lower molecular weight. The novel peptides can be schematically depicted as follows:

---------A------[----------X----------]--------B---------

wherein region A corresponds to a sufficient number of amino acids 20-759 of Fig. 1 and region B corresponds to a sufficient number of amino acids 1709-2351 of Figure 1 to provide procoagulant activity; and region X represents a polypeptide sequence comprising from 1 to 700 amino acids which will not significantly reduce the combined procoagulant activity of regions A and B

The 1 to 700 amino acids that are represented by X should be selected so that they do not substantially affect the procoagulant activity of the molecule. Preferably, the amino acids correspond to sequences of amino acids 760-1708 of Figure 1. More preferably, the region represented by X contains 100-400 amino acids.

The procoagulant protein may be produced by appropriate host cells transformed by factor VIII:C DNA which has been specifically altered by use of any of a variety of site-specific mutagenesis techniques which will be familiar to those of ordinary skill in the art of recombinant DNA.

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The starting materials may be a DNA sequence which codes for the complete factor VIII:C molecule, e.g., the complete human factor VIII:C as shown in Figure 1, a truncated version of that sequence, or it may comprise segments of that DNA sequence, so long as the starting materials contain at least sufficient DNA to code for the amino acid sequences of the desired polypeptide.

One of the problems presently encountered with the use of human factor VIII:C for treatment of hemophilia arises from its antigenicity. A significant percentage of hemophiliacs have developed an immune reaction to the factor VIII:C used for their treatment. Non-hemophiliacs can also develop or acquire hemophilia when their immune systems become sensitized to factor VIII:C and produce circulating antibodies or "inhibitors" to factor VIII:C. In either case, the effect is the neutralization of whatever factor VIII:C is present in the patient, making treatment very difficult. Until now, the method of choice for treating hemophiliacs with this problem has been to administer, in cases of severe bleeding episodes, non-human factor VIII:C, such as treated porcine factor VIII:C. See Kernoff et al., Blood 63:31 (1984). However, the antibodies which neutralize the clotting ability of human factor VIII:C will react to a varying extent with factor VIII:C of other species, and the porcine protein is itself antigenic, thus both the short-term and long-term effectiveness of such treatment will vary. Additionally, patients frequently display adverse reactions to

infusion with the porcine factor VIII:C. The use of porcine factor VIII:C in spite of the risks has been justified because of the lack of reliably effective alternatives. Kernoff, supra at 38. The present invention provides an alternative to the administration of porcine factor VIII:C.

The procoagulant proteins of the present invention, in addition to lacking a substantial amino acid segment of human factor VIII:C, also have fewer potential N-glycosylation sites than human factor VIII. Preferably, at least one N-glycosylation site has been deleted. More preferably, 18 of the 25 potentional N-glycosylation sites are not in the molecule. In still more preferred embodiments, up to 19 of the 25 potential N-glycosylation sites are removed. While not wanting to be bound by theory, it is believed that the antibodies to factor VIII:C which are directed to antigenic determinants contained in the amino acid segment deleted in accordance with this invention; i.e., in the amino acid segment itself or in the carbohydrate portion of the glycosylated protein, will not neutralize the procoagulant proteins of the present invention. Moreover, the fact that the procoagulants of the present invention lack many of the sites for non-human glycosylation by the non-human mammalian or other cells used to produce the proteins is also believed to reduce the antigenicity of that protein, and lessen the likelihood of developing antibodies to these procoagulants.

This may enable facilitating the treatment of patients in need of procoagulant therapy.

I contemplate that my compounds can be produced by recombinant DNA techniques at a much lower cost than is possible for production of human factor VIII. The host organism should more efficiently process and express the substantially simpler molecules of this invention.

One method by which these polypeptides can be expressed is by use of DNA which is prepared by cutting a full-length factor VIII:C DNA with the appropriate restriction enzymes to remove a portion of the DNA sequence that codes for amino acids 760 to 1708 of human factor VIII:C. The cut DNA is then ligated with an oligonucleotide that resects the cut DNA and maintains the correct translational reading frame.

Preparation of the cDNA has been set forth in detail in U.S. Patent Applications Serial Nos. 546,650 and 644,086, supra. A pSP64 recombinant clone containing the nucleotide sequence depicted in Figure 1, designated as pSP64-VIII, is on deposit at the American Type Culture Collection under Accession Number ATCC 39812.

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Restriction endonucleases are used to obtain cleavage of the human factor VIII:C cDNA, hereinafter the DNA source sequence, at appropriate sites in the nucleotide sequence. Unless otherwise noted, restriction endonucleases are utilized under the conditions and in the manner recommended by their commericial suppliers. The restriction endonucleases selected herein are those which will enable one to excise with substantial specificity sequences that code for the portion of the factor VIII:C molecule desired to be excised. BamHI and SacI are particularly useful endonucleases. However, the skilled artisan will be able to utilize other restriction endonucleases chosen by conventional selection methods. The number of nucleotides deleted may vary but care whould be taken to insure that the reading frame of the ultimate cDNA sequence will not be affected.

The resulting DNA fragments are then purified using conventional techniques such as those set forth in Maniatis et al., Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory 1982) the disclosure of which is incorporated herein by reference, and Proc. Natl. Acad. Sci. 76:615-619 (1979). The purified DNA is then ligated to form the sequence encoding the polypeptide of the preferred invention. When necessary or desirable, the ligation may be with an oligonucleotide that resects the cut DNA and maintains the correct translational reading frame using standard ligation conditions. Ligation

reactions are carried on as described by Maniatis et al., supra at 2453-6 using the buffer described at page 246 thereof and using a DNA concentration of 1-100 ug/ml, at a temperature of 23°C for blunt ended DNA and 16°C for "sticky ended" DNA. The following double-stranded oligonucleotide is useful when there is BamHI/SacI deletion such as described infra,

5' P-CATGGACCG-3'
3-TCGAGTACCTGGCCTAG 5';

but other oligonucleotides can be selected by the skilled artisan depending upon the deletions made and reaction conditions.

The DNA sequences encoding the novel procoagulant polypeptides can, in addition to other methods, be derived form the sequence of human factor VIII:C DNA by application of oligonucleotide-mediated deletion mutagenesis, often referred to as "loopout" mutagenesis, as described for example in Morinaga, Y. et al. Biotech. 2:636-639 (1984).

The new DNA sequences containing the various deletions can then be introduced into appropriate vectors for expression in mammalian cells. The procoagulant activity produced by the transiently transfected or stably transformed host cells may be measured by using standard assays for blood plasma samples.

The eucaryotic cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors such as the bacterial replicons, selection genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized by known procedures. See Kaufman et al., J. Mol. Biol., 159:51-521 (1982); Kaufman, Proc. Natl. Acad. Sci. 82:689-693 (1985).

Established cell lines, including transformed cell lines, are suitable as hosts. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants (including relatively undifferentiated cells such as haematopoeitic stem cells) are also suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting.

The host cells preferably will be established mammalian cell lines. For stable integration of the vector DNA into chromosomal DNA, and for subsequent amplification of the integrated vector DNA, CHO (Chinese hamster ovary) cells are best. See U.S. Patent 4,399,216. Alternatively, the vector DNA could include all or parts of the bovine papilloma virus genome (Lusky et al., Cell, 36:391-401 (1984)) and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other usable mammalian cell lines include HeLa, COS-1 monkey cells, melanoma cell lines such as Bowes cells, mouse L-929 cells, 3T3 lines derived from

Swiss, Balb-c or NIH mice, BHK or HaK hamster cells lines and the like.

Stable transformants then are screened for expression of the procoagulant product by standard immunological or enzymatic assays. The presence of the DNA encoding the procoagulant proteins may be detected by standard procedures such as Southern blotting. Transient expression of the procoagulant genes during the several days after introduction of the expression vector DNA into suitable host cells such COS-1 monkey cells is measured without selection by enzymatic or immunologic assay of the proteins in the culture medium.

The invention will be further understood with reference to the following illustrative embodiments, which are purely exemplary, and should not be taken as limiting the true scope of the present invention, as described in the claims.

EXAMPLE 1

10 ug of the plasmid pACE, an pSP64 (Promega Biotec, Madison, Wis.) derivative, containing nucleotides 562-7269 of human factor VIII:C cDNA (nucleotide 1 is the A of the ATG initiator methionine codon) was subjected to partial BamHI digestion in 100ul containing 50mM Tris.HCl pH 8.0, 50 mM $MgCl_2$, and 2.4 units BamHI (New England Biolabs) for 30 minutes at 37°C. The

reaction was terminated by the addition of EDTA to 20mM and then extracted once with phenol, once with choloroform, ethanol precipitated and pelleted by centrifugation. DNA was redissolved, cleaved to completion in 50ul using 40 units SacI for 1.5 hours at 37°C. DNA was then electrophoresed through a buffered 0.6% agarose gel. An 8.1 kb fragment corresponding to the partial BamHI-SacI fragment of pACE lacking only the sequence corresponding to nucleotides 2992-4774 of the factor VIII:C sequence was purified from the gel using the glass powder technique described in Proc. Nat. Acad. Sci. 76:615-619 (1979). Purified DNA was ligated with 100 pmoles of the following double-stranded oligonucleotide

5' P-CATGGACCG-3'
3-TCGAGTACCTGGCCTAG 5'

using standard ligation conditions. The DNA sequence removed represents the deletion of a 584 amino acid sequence beginning with amino acid 998 and continuing through 1581. The oligonucleotide inserted, however, encodes amino acids corresponding to 998-1000. Therefore, the polypeptide encoded contains a deletion of 581 amino acids.

DNA was then used to transform competent E. coli bacteria and DNA from several ampicillin resistant transformants was analyzed by restriction mapping to identify a plasmid harboring the desired SacI-BamHI deletion mutant. DNA from this plasmid was

digested to completion with KpnI, which cleaves the plasmid uniquely at nucleotide 1816 of the factor VIII:C coding sequence. This DNA was ligated with a KpnI DNA fragment containing nucleotides 1-1815 of factor VIII:C DNA and a synthetic SalI site at nucleotides -11 to -5 and then used to transform competent E. coli bacteria.

Plasmid DNA was isolated and oriented by restriction mapping to identify a plasmid, pBSdK, containing the correct 5' to 3' orientation of the KpnI insert. SalI digestion, which excises the entire polypeptide coding region from the plasmid, was performed and the DNA electrophoresed through a buffered 0.6% agarose gel. The 5.3Kb SalI fragment was purified from the gel as described above. This DNA fragment was ligated with XhoI cut pXMT2 DNA to give rise to plasmid pDGR-2. pXMT2 is a plasmid capable of expressing heterologous genes when introduced into mammalian cells such as the COS-1 African Green Monkey kidney cell line, and is a derivative of the expression vectors described in Kaufman, supra at 689-93. The expression elements are the same as described for plasmid pD61. The bacterial replicon, however, has been substituted to render bacteria containing the vector resistant to ampicillin rather than tetracycline. pXMT2 contains a unique Xho I site at a position which allows for expression of inserted cDNA from the adenovirus major late promoter. This Xho I site is convenient for inserting factor VIII:C cDNA constructs since these are flanked by SalI sites.

Restriction mapping of transformants identified a plasmid, pDGR-2, containing the correct 5' to 3' orientation of the polypeptide coding sequence relative to the direction of transcription from the adenovirus major late promoter. pDGR-2 is on deposit at the American Type Culture Collection under Accession number --------. pDGR-2 was introduced into the COS-1 cell line via the DEAE-dextran transfection protocol and conditioned media assayed for factor VIII:C type procoagulant activity as described in Toole et al., supra Nature at 326. Procoagulant activity of about 19 mU/ml was obtained compared with about 4 mU/ml obtained from pXMT2 containing the complete factor VIII:C cDNA.

Example 2

Other novel procoagulant proteins may be obtained from constructs produced by oligonucleotide mediated deletion mutagenesis, using for example the "loopout" mutagenesis techniques as described in Morinaga et al., supra. The deletion mutagenesis is performed using expression plasmid pDGR-2 or any other appropriate plasmid or bacteriophage vector. Other methods for oligonucleotide mediated mutagenesis employing single stranded DNA produced with M13 vectors and the like are also suitable. See Zoller et al., Nucl. Acids Res. 10:6487-6500 (1982).

For example, these deletions can be produced using the oligonucleotides.

(A) 5' AAAAGCAATTTAATGCCACCCCACCAGTCTTGAAACGCCA

(B) 5' AAAAGCAATTTAATGCCACCGAAGATTTTGACATTTATGA

to cause deletions in factor VIII:C cDNA from nucleotides (A) 2334 to 4974 or (B) 2334 to 5079. The proteins encoded by these constructs would delete (A) 880 and (B) 915 amino acids.

The deleted constructs are tested directly, or after subcloning into appropriate expression vectors, in order to determine if the novel proteins possess procoagulant activity. Procoagulant activity is assayed as described in Example 1.

In accordance with another aspect of this invention, there is provided a pharmaceutical preparation of the novel polypeptide in a parenterally acceptable vehicle which may be prepared in accordance with procedures well known in the art.

The compounds of this invention can be formulated into pharmaceutically acceptable preparations with parenterally acceptable vehicles and excipients in accordance with procedures well known in the art.

The pharaceutical preparation of this invention, suitable for

parenteral administration, may conveniently comprise a sterile lyophilized preparation of the polypeptide which may be reconstituted by addition of sterilized solution to produce solutions preferably isotonic with the blood of the recipient. The preparation may be presented in unit or multi-dose containers, for example sealed ampoules or vials. Their use would be analagous to that of human factor VIII appropriately adjusted for potency.